

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>A01H 4/00, A61K 39/00, C07K 14/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/08375</b> <b>(43) International Publication Date:</b> 5 March 1998 (05.03.98)
<b>(21) International Application Number:</b> PCT/US97/15200 <b>(22) International Filing Date:</b> 28 August 1997 (28.08.97)  <b>(30) Priority Data:</b> 08/704,856 28 August 1996 (28.08.96) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/704,856 (CIP) Filed on 28 August 1996 (28.08.96)  <b>(71) Applicant (for all designated States except US):</b> THOMAS JEFFERSON UNIVERSITY [US/US]; 1020 Walnut Street, Philadelphia, PA 19107 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KOPROWSKI, Hilary [US/US]; 334 Fairhill Road, Wynnewood, PA 19096 (US). HOOPER, Douglas, Craig [CA/US]; 2 Oregon Trail, Medford, NJ 08055 (US). YUSIBOV, Vidadi [RU/US]; Apartment 512, 2323 East Darby Road, Havertown, PA 19083 (US). MODELSKA, Anna [PL/US]; 334 Fairhill Road, Wynnewood, PA 19096 (US).		<b>(74) Agents:</b> VOLPE, Anthony, S. et al.; Volpe and Koenig, P.C., 400 One Penn Center, 1617 John F. Kennedy Boulevard, Philadelphia, PA 19103 (US).  <b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> POLYPEPTIDES FUSED WITH ALFALFA MOSAIC VIRUS OR ILARVIRUS CAPSID  <b>(57) Abstract</b> <p>A fusion capsid protein comprising a plant virus capsid protein fused to an antigenic polypeptide is used as a molecule for presentation of that polypeptide to the immune system of an animal such as a human. The plant virus capsid protein is that of an alfalfa mosaic virus (AIMV) or ilarvirus.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

WU 50/05/75 FC 1/057/115200

## POLYPEPTIDES FUSED WITH ALFALFA MOSAIC VIRUS OR ILARVIRUS CAPSID

### FIELD OF THE INVENTION

The field of the invention is recombinant plant viruses, especially their use as immunizing agents which carry  
10 antigenic sequences from mammalian (e.g., human) or other animal pathogens and their use as a system for increased production of polypeptides of interest.

### BACKGROUND OF THE INVENTION

Traditionally, successful vaccination has been dependent  
15 upon the use of live attenuated viruses or preparations of killed pathogenic organisms. These vaccines are very effective in controlling or, as in the case of smallpox, even eliminating certain infectious diseases. However, their use often present safety concerns. Subunit vaccines based on  
20 peptide or proteins derived from a pathogen are less hazardous than traditional vaccines but have generally suffered from poor immunogenicity and high expense. Moreover, current vaccines with a few exceptions must be administered parenterally. However, it is well known that most pathogens  
25 gain entry across the mucosal surfaces of the body and a mucosal immune response would therefore be more appropriate.

Both safety concerns and the desire to target mucosal tissues for more effective immunization against common pathogens dictate the need for new approaches to vaccination.

-2-

For induction of a mucosal response, oral administration of antigen is appropriate, inexpensive, and safe. However, in order to efficiently immunize by the oral route, several obstacles such as degradation from low pH or proteases in the gastrointestinal GI tract, the short exposure to immune induction sites, and limited permeability must be overcome.

Recent studies demonstrate that plants and plant viruses can function as effective tools for vaccine production and delivery. Furthermore, like liposomes and microcapsules, it is expected that plant cells and plant viruses will serve as delivery vehicles providing natural protection for the antigen associated with them and enhancing the uptake of the antigen from the GI tract. Such new developing "green system vaccines" have significant advantages over the traditional and synthetic vaccines as regards safety, deliverability via either parenteral, nasal or oral routes, and lower cost of production.

#### BRIEF SUMMARY OF THE INVENTION

In one general aspect, the current invention is a process of delivering a fusion capsid protein (a plant virus capsid protein fused to a foreign polypeptide) to a mammal (such as a human) or other animal using recombinant tobacco mosaic virus (TMV) genetic material (TMV genetic material combined with genetic material that codes for a chimeric capsid protein, the chimeric capsid protein being capsid protein of either an alfalfa mosaic virus (AlMV) capsid protein (CP) or ilarvirus CP, fused to the foreign polypeptide) as a delivery

-3-

vehicle to that mammal or other animal. A foreign polypeptide is one that does not naturally occur in either TMV, an AlMV or an ilarvirus. The fusion protein is administered to the mammal or other animal for purposes of inducing an immune response against the foreign polypeptide. In a second general aspect, the invention is a production process: the use of such a chimeric virus to express the fused coat protein (comprising either an antigenic or nonantigenic foreign protein) in a plant.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1A.** Schematic representation of the genome of TMV. The regions of the genome coding for the 126 kDa and 183 kDa proteins required for virus replication, the 30 kDa viral movement protein, and the CP (viral coat protein) are shown schematically. The arrow under "TMV CP SP" indicates the subgenomic promoter of TMV. The three connected ellipsoids under "pep" represent the polypeptide fused to the AlMV CP. Rz- indicates ribozyme. B3ORz- is a derivative of TMV. AvB3ORz- is a derivative of B3ORz and is defective as to translation of coat protein.

**Figure 1B.** A schematic representation of cloning strategy: the cloning of chimeric AlMV CP into a TMV based vector. The sequences of polypeptides from HIV-1 and rabies virus are used as the "pep" to create recombinant viruses.

The "pep" polypeptide has the following amino sequence:  
For BrzCPMNV3: CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC (SEQ ID NO:1)

-4-

For BRzCPNLV3: CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC (SEQ ID NO:2)

For BRzCPDnv10c: MSAVYTRIMMNGGRLKRYEAAELTLTDVALADDS (SEQ ID NO:3)

5 For BRzCPDrg24: MSAVYTRIMMNGGRLKRPPDQLVALHDGIEKLVVEEDS (SEQ ID NO:4)

For BRzCPNLpr:

10 HIV-1 NL 4.3 Vpr-  
MEQAPEDQGPREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQHIYETYGDTW  
AGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS  
(SEQ ID NO:15)

For BRzCPNLVpu:

15 HIV-1 NL 4.3 Vpu-  
MQPIIIVAIVALVVAIIIAIVVWSIVIIIEYRKILRQRKIDRLIDRLIERAEDSGN  
ESEGEVSALVEMGVEMGHHPWDIDDL (SEQ ID NO:16)

Figure 2. Accumulation of chimeric AlMV CP, fused with different peptides, in tobacco protoplasts infected with transcripts of recombinant virus. Proteins were separated by electrophoresis in a 13% SDS-polyacrylamide gel and electroblotted on nylon membrane. The proteins were reacted with monoclonal antibodies to AlMV CP followed by detection with Westatin immunostain kit (Sigma). Lane 1 represents wt AlMV CP. Lane 2 and 3 are *in vitro* translation products of pSPCPD10c and pSPCPDrg24, respectively. Lane 4- pBRzCPNLVpu, lane 5-pBRzCPNLVpr, lane 6-pBRzCPDrg24, lane 7-pBRzCPDNV10c, lane 8-pBRzCPMNV3, lane 9-pBRzCPNLV3, and lane 10-B30Rz.

Figure 3. Electron micrographs of recombinant AlMV particles from tobacco plants, infected with recombinant transcripts of TMV presenting different constructs. The

-5-

particles were negatively stained with 2% of uranyl acetate. The bars indicate 100 nm. A-B30Rz. B- pBRzCPNLV3. The single arrow indicates a TMV particle. The double arrow indicates indicates recombinant AllMV particles.

5           **Figure 4.** Accumulation of chimeric AIMV CP in systemically infected tobacco leaves. The tobacco leaves were inoculated with transcripts of recombinant virus. Proteins were separated by electrophoresis in a 13% SDS-polyacrilamide gel. The proteins were reacted with monoclonal  
10 antibodies to AIMV CP followed by detection with Westatin immunostain kit. Lane 1 represents wt AIMV CP. Two- pBRzCPNLVpu, 3- B30Rz, 4- pBRzCPDrg24, 5- pBRzCPDNV10c, 6- pBRzCPNLVpr, 7- pBRzCPMNV3, and 8- pBRzCPNLV3.

15           **Figure 5.** Immunoprecipitation of chimeric particles containing rabies and HIV-1 epitodes. The particles purified from plant tissue which were coinfectd with transcripts of recombinant virus and immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24). Immunoprecipitated proteins were separated by electrophoresis  
20 in a 13% SDS-polyacrilamide gel. The proteins were reacted with antibodies to HIV-1 V3 loop or with monoclonal antibodies to N protein of rabies followed by detection with Westatin immunostain kit. Lane 1 represents immunoprecipitated proteins reacting with antibodies to N protein. Two- wild  
25 type rabies. 3- HIV-1 gp120 reacted with antibodies to V3 loop, and 4- immunoprecipitated proteins reacting with antibodies to V3 loop.

-6-

**Figure 6A and B.** Results obtained in ELISA and neutralization assay as a comparison of control mouse immunized with mix of TMV and AIMV and mouse immunized with pBRzCPDrg24. Eight-week-old female Swiss-Webster outbred mice were immunized with 10-ug doses of each recombinant virus engineered to express epitopes. Three immunizations of 0.1 mL were administered intraperitoneally at 2-week intervals with complete Freund's adjuvant at a 1:1 (v:v) ratio. An equal quantity of a mixture of wild type TMV plus AIMV was used with complete Freund's adjuvant as a control. Ten to 14 days after each immunization, serum samples were obtained from individual mice, and specific antibody titers for rabies virus were assessed using a solid-phase enzyme-linked immunosorbent assay. Assay results are expressed in O.D. units. Specific neutralization of rabies CVS-11 virus was assessed in a modified rapid fluoresescent focus-forming assay using serum from pBRzCPDrg24-immunized mice and BHK indicator cells. One out of five mice had neutralizing antibodies.

**Figure 7.** The precentage of mice producing specific rabies virus antibodies after I. p. immunization with pBRzCPDrg24 (Rg24-A/TMV) or mix of AIMV plus TMV (Control). The results were obtained in ELISA. Experiment is described in the legend to **Figure 6**.

**Figure 8.** Serum antibody response of mice immunized with CPMNV3 and neutralizing activity of those antibodies. Serum antibody response was measured by ELISA on plates coated with synthetic peptide resembling the V3 loop of HIV-1. **Fig. 8A** indicates the serum antibody response when the immunogen



-7-

was administered without CFA whereas in Fig. 8B CFA was added. Also shown are the ELISA data for preimmune and mice immunized with control virus. Fig. 8C represents neutralization of HIV-1/MN isolate by sera from mice immunized with CPMNV3. Data points in Fig. 7C are averages obtained using preimmune and sera after the last (seventh) inoculation of antigen.

Figure 9. Serum antibody response of mice immunized with CPDrg24. Serum antibody response was measured by ELISA using a 1:40 dilution of serum and plates coated with AlMV (vector plates) or inactivated ERA strain rabies virus (ERA plates). Fig. 9A shows the serum IgA response specific for vector and Drg24 whereas in Fig. 9B shows the serum IgG response specific for vector and Drg24. Also shown are the ELISA data for preimmune and mice immunized with control virus.

#### DETAILED DESCRIPTION

##### GLOSSARY AND DISCUSSION OF TERMS USED

A "plant" for purposes of this patent application includes liverworts (Hepaticae), mosses (Musci), psilopsids (Psilopsida), club mosses (Lycopsidea), horsetails (Sphenopsida), ferns and seed plants, and certain fungi specified below, and algae including blue-green algae. Ferns and seed plants together make up the Pteropsida. Seed plants include gymnosperms (Gymnospermae) and angiosperms (Angiospermae). The great majority of plants used for food are angiosperms. For purposes of this application, the following fungi are considered plants: Basidiomycetes, which include

mushrooms. The following are not considered plants for purposes of this application: bacteria, single-celled eukaryotes, and the following fungi: Phycomycetes, Ascomycetes (yeasts), and Deuteromycetes.

5           The term "plant tissue" includes any tissue of a plant. Included are whole plants, any part of plants, plant cells, plants seeds, and plant protoplasts.

          The word "animal" refers to humans as well as other animals.

10           A "bird" is a warm-blooded vertebrate of the class Aves.

          A "fish" is a cold-blooded aquatic vertebrate, having gills and fins.

          A "chimeric protein" is created when two or more genes that normally code for two separate proteins recombine, either  
15           naturally or as the result of human intervention, to code for a protein that is a combination of all or part of each of those two proteins.

          The phrase "code for" is used in this application to refer to both the nucleic acid sequence that codes for a  
20           polypeptide sequence and a base sequence complementary to such a nucleic acid sequence.

          A "fusion capsid protein" is a chimeric protein in which one of the genes in the chimera codes for a plant virus capsid protein.

25           A "bipartite fusion capsid protein" is a fusion wherein genes for two proteins recombine.

          A "tripartite fusion capsid protein" is a fusion wherein genes for three proteins recombine.

A "pathogen protein" is a protein that is coded for by the genetic material of a pathogen.

A "naturally occurring plant protein" is one that is normally found in a plant, at least one stage in its life cycle, in its natural habitat.

"Infecting a plant cell" means infecting a plant cell with one or more genes that it does not naturally have, the infection being by a nucleic acid molecule that may or may not be encapsidated within a virus.

"Protective immunity" is the ability of an animal, such as a mammal, bird, or fish, to resist (delayed onset of symptoms or reduced severity of symptoms), as the result of its exposure to the antigen of a pathogen, disease or death that otherwise follows contact with the pathogen. Protective immunity is achieved by one or more of the following mechanisms: mucosal, humoral, or cellular immunity. Mucosal immunity is primarily the result of secretory IgA (sIGA) antibodies on mucosal surfaces of the respiratory, gastrointestinal, and genitourinary tracts. The sIGA antibodies are generated after a series of events mediated by antigen-processing cells, B and T lymphocytes, that result in sIGA production by B lymphocytes on mucosa-lined tissues of the body. Mucosal immunity can be stimulated by an oral vaccine. The primary result of protective immunity is the destruction of the pathogen or inhibition of its ability to replicate itself.

"Humoral immunity" is the result of IgG antibodies and IgM antibodies in serum.

"Cellular immunity" can be achieved through cytotoxic T lymphocytes or through delayed-type hypersensitivity that involves macrophages and T lymphocytes, as well as other mechanisms involving T cells without a requirement for antibodies. A "derivative cell" derived from an infected plant cell is one created as a result of the infected plant cell undergoing cell division or a series of cell divisions such that one or more copies of the foreign gene introduced into the plant cell by infection is in the derivative cell.

"Ilarviruses" includes the following subgroups: tobacco streak virus, prune dwarf virus, lilac ring mottle virus, citrus leaf rugose virus, citrus variegation virus, elm mottle virus, spinach latent virus, asparagus virus 2, Parietaria mottle virus, hydrangea mosaic virus, apple mosaic virus, Prunus necrotic ringspot virus, tulare apple mosaic virus, blueberry scorch virus, cherry rugose virus, danish plum line pattern virus, Hop A virus, Hop C virus, American plum line pattern virus, and Humulus japonicus virus.

A "recombinant virus" is one in which the genetic material of a virus has combined with other genetic material.

A "polypeptide" is a molecule in which there is at least four amino acids linked by peptide bonds.

"Viral nucleic acid" may be the genome (or the majority thereof) of a virus, or a nucleic acid molecule complementary in base sequence to that genome. A DNA molecule that is complementary to viral RNA is also considered viral nucleic acid. An RNA molecule that is complementary in base sequence to viral DNA is also considered to be viral nucleic acid.

-11-

"AlMV" is alfalfa mosaic virus.

"TMV" is tobacco mosaic virus.

5 A "vaccine" in the present invention is the fusion capsid protein, any particle of which that protein is part, or any preparation such as plant material of which that protein is part.

"Plurality" means more than one.

#### ASPECTS OF THE INVENTION

10 In a general aspect, the invention is a process of administering a polypeptide to an animal (especially a mammal, bird, or fish), the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed in the plant cell to produce a fusion capsid protein comprising virus capsid  
15 protein and a polypeptide that is not a plant virus capsid protein, said virus capsid protein being an AlMV coat protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said  
20 infected cell or derivative cell makes the fusion capsid protein; and

(3) administering the fusion capsid protein or a portion thereof to an animal.

25 In step (1) the viral nucleic acid may either be in a virus or not in a virus. If it is not in a virus, it may either be pure nucleic acid or nucleic acid associated with other molecules or molecular structures.

-12-

In step (1) if the recombinant plant virus nucleic acid comprises TMV nucleic acid, the TMV nucleic acid will preferably comprise nucleic acid coding for the TMV 123 kDa 183 kDa, and 30 kDa proteins.

5 In steps (1) and (2), the infected cell or derivative cell may either be part of a plant or plant tissue or may be free of other plant cells.

In step (1), it is preferable that the viral nucleic acid have sufficient genetic information to produce viral particles  
10 during step (2).

The route of administration in step (3) can be parenteral or nonparenteral. If administered parenterally, the protein to be administered to the animal will preferably be substantially pure of other material found in the plant cells  
15 that produced it .

In step (3), the protein or portion thereof may be in or part of the infected or derivative cell, part of an extract of such a infected or derivative cell or, as a result of protein purification, free of other material normally present  
20 in the infected or derivative cell.

The result of step (3) is, in a particular embodiment of the invention, an immune response against the part of the fusion capsid protein that is not plant viral protein. That immune response preferably results in either protective  
25 immunity or systemic tolerance.

The steps for purifying a fusion capsid protein are ones commonly used for the fractionation of plants into their protein components and the separation of individual proteins

from other components of the infected or derivative cell. Such steps include protection of the native conformation of the pathogen protein by steps such as flash freezing the plant material with dry ice or liquid nitrogen.

5           An extract would be created by a process comprising mechanical or chemical disruption of a cell. In some cases, additional protein purification steps would be used to make the extract.

10           In one important embodiment of the invention, in step (3) the fusion capsid protein is in part of a plant or plant product and is fed to the mammal (i.e. oral route of administration). In such a case, it is preferable that the plant is raw; i.e., has not been cooked (heated above the temperatures associated with growth, storage, and transport).  
15           In the examples described below, the plant is not cooked. Animals typically may eat the plant, pieces of the plant, a puree from the plant, or plant juice. As a result, it is frequently preferred that step (2) takes place in an edible plant or part of an edible plant.

20           Animals vary as regards which food is edible. Plants of greatest interest include potatoes, tomatoes, peas, beans, alfalfa, citrus fruits (e.g., oranges, lemons, grapefruit), grapes, carrots, strawberries, blueberries and other berries, bananas, rice, wheat, corn, barley, oats, rye, dates, cabbage,  
25           Brussel sprouts, cauliflower, turnips, cucurbits, papaya, guava, apples, cherries, apricots, pears, and grapes.

          In another important embodiment of the invention, the fusion capsid protein of interest is extracted in purified

-14-

form from the plant and administered as a substantially pure protein, possibly with an adjuvant or other compounds needed to facilitate or improve vaccine administration.

As in the case of the rabies virus glycoprotein (Rgp), the polypeptide referred to in step (1) may be one that is a glycoprotein in the pathogen.

#### Pathogens of interest

A pathogen is any organism such as a virus, bacterium, fungus, or parasite, as well as a protein which is capable of self-replication such as a prion and capable of inducing disease in an animal.

Pathogens against which vaccines created by the present invention are effective are those including but not limited to bacteria of the genera streptococci and staphylococci, as well as the mycoplasma, rickettsia and spirochetes. The following viral groups of the parvoviridae, papovaviridae, adenoviridae, herpesviridae, poxviridae, iridoviridae, picornaviridae, caliciviridae, togaviridae, flaviviridae, coronaviridae, ortho- and paramyxoviridae, rhabdoviridae, bunyaviridae, reoviridae, birnaviridae, and the retroviruses all contain representative members with application for vaccines of the present invention.

Pathogens against which vaccines of the present invention are expected to be particularly useful are rabies, respiratory syncytial virus, cholera, typhoid fever, herpes simplex types I and II, tuberculosis, pathogenic pneumococci, human immunodeficiency virus-1 (HIV-1) and human immunodeficiency



-15-

virus-2 (HIV-2). The spectrum of pathogens includes those of veterinary significance and includes parvoviridae, papovaviridae, adenoviridae, herpesviridae, poxviridae, iridoviridae, picornaviridae, caliciviridae, togaviridae, flaviviridae, coronaviridae, ortho- and paramyxoviridae, rhabdoviridae, bunyaviridae, reoviridae, birnaviridae, and the retroviruses. Both gram negative and gram positive bacteria and spirochetes are also expected to be pathogens which may be clinically affected by the plant vaccines of the present invention.

#### Methods for infecting plants

Genes may be transferred by any of a variety of infection means, which means include but are not limited to:

1). Coating a plant surface with viruses or viral nucleic acid, possibly one that has undergone abrasion;

2) Microparticle bombardment, for example as described for wheat by V. Vasil et al., *Bio/Technology* 9, 743 (1991) and generally in *Bio/Technology* 10, 286 (1992).

3) Electroporation, for example as described for lettuce by M. C. Chupeau et al., *Bio/Technology* 7, 503 (1989).

4) Liposome fusion with protoplasts (A. Deshayes et al., *EMBO J.* 4, p2731-2737 (1985)).

5) Polyethylene glycol-mediated transformation (I. Potrykus et al., *Mol. Gen. Genetics*, 197, 183-188).

6) Microinjection (R. Griesbach, *Biotechnology* 3, p348-350; C.K. Shewmaker *Mol. Gen. Genetics*, 202 p. 179-185 (1986)).

It may be desirable to target protein expression to different plant tissues (i.e. fruit and leaves) and to different intracellular locations (i.e. chloroplast, vacuole, plasma membrane) in order to enhance protection of the recombinant protein. It may also be desirable to present the antigen as part of larger structure for uptake by the M cells of the Peyers patch.

It may be desirable to combine presentation of the primary antigens with a biologically active molecule that will stimulate and or enhance an immune response and serve as an adjuvant. Since modified plants can be crossed genetically, plants expressing an adjuvant protein and a primary antigen can be produced separately and combined to deliver antigen and adjuvant in one plant.

#### Plant viruses

The quasi-isometric/bacilliform viruses can, according to J. A. Levy et al. (Virology, 3rd edition, Prentice Hall, Englewood Cliffs, New Jersey (1994)), be divided into two subgroups: AlMV and the ilarviruses. The antigen-presenting capsid protein in the present invention is that of a quasi/bacilliform virus: its synthesis is driven by a genome constructed to produce a chimeric capsid protein whose components include the quasi/bacilliform capsid protein and the antigenic protein or polypeptide of interest. The virus that delivers the chimeric capsid protein to a plant for production of the antigenic protein may itself be a quasi-isometric/bacilliform virus, or alternatively, may be a

nonenveloped virus with a positive-sense RNA genome that is not a quasi-isometric/bacilliform virus. Below, examples are given of how AlMV or TMV can form the major part of the delivery vehicle.

5 Tobacco mosaic virus and alfalfa mosaic virus

Two viruses used herein to exemplify the invention are tobacco mosaic virus (TMV) and alfalfa mosaic virus (AlMV). TMV is the type member of the tobamovirus group. TMV consists of a single plus-sense genomic RNA (6.5 kb) encapsidated with  
10 a unique coat protein (17.5 kDa) which results in rod-shaped particles (300 nm). A wide host range of tobacco mosaic virus allows one to use a variety of plant species as production and delivery systems. The expression of protein depends only upon virus replication and does not interfere with plant nuclear  
15 transcription machinery thus providing high levels of protein accumulation in infected cells. It has been shown that foreign genes inserted into this vector can produce high levels of protein (Yusibov et al., *Proc. Natl. Acad. Sci. U.S.* 92, 8980 (1995)). The second component of a recombinant expression  
20 vector exemplified herein is AlMV CP. AlMV is a type member of *Bromoviridae* family. The genome of this virus consists of three positive-sense RNAs (RNAs 1-3) which are encapsidated by CP (24 kDa) resulting in a 30-60 nm bacilliform particle. The fourth RNA (subgenomic RNA4) of AlMV is the messenger for  
25 CP and is synthesized from genomic RNA3. RNA4 is separately encapsidated into spherical particles of 20, 30 nm. The

-18-

functions of CP thought to be important for the virus include: initiation of infection, stability of viral RNA, switching from minus to plus RNA, movement of the infectious material throughout the host, and encapsulation. The N-terminus of the

5 AlMV CP specifically binds to the 3' noncoding region of all AlMV RNAs, requiring the AUGC repeats at the 3' end of viral RNAs and initiates the infection. The carboxyterminus of CP is required for the encapsidation of viral RNA. Earlier we have shown that AlMV CP carrying 37 amino acids at its N-

10 terminus will preserve the biological activity and form the particles *in vitro* (Yusibov et al., J. Gen. Virol 77, 567 (1996)). Because of the ability of AlMV CP to form these particles *in vitro* and carry large peptides, the AlMV CP is used as an additional component to complement TMV expression

15 system. *In vitro* transcripts of TMV carrying inserted sequences are used for the mechanical inoculation of tobacco plants. Within 10-14 days after inoculation, infected plant tissue can be used to determine the amount of protein in tissue, for purification of virus particles, and for the

20 feeding experiments to test mucosal immunogenicity. For the latter, low alkaloid tobacco plants that can be fed to the animals are used to grow the recombinant viruses.

After infection with recombinant viral RNA-coding for a fusion protein (AlMV capsid protein fused to an antigenic

25 polypeptide), the infecting RNA is processed and the infection results in two types of viral particles:

- 1) recombinant TMV-recombinant viral RNA encapsidated with wild type TMV CP;

-19-

2) recombinant AlMV-non infectious AlMV particles consisting of subgenomic RNA coding for fusion protein (AlMV capsid protein fused to an antigenic polypeptide) and fusion protein.

5 The particles consisting of recombinant AlMV CP serve as antigen delivery and presentation systems. These particles have advantages over recombinant TMV particles that do not have AlMV or ilarvirus fusion capsid proteins. The use of AlMV or ilarvirus capsid protein allows the assembly of up to  
10 100 amino acids presenting foreign antigenic or nonantigenic sequences into particles for effective antigen presentation. The TMV CP is capable presenting up to 25 amino acids. The larger peptides interferes with TMV assembly. Additionally, AlMV CP molecules each of which is fused to  
15 different antigenic polypeptides will assemble into multivalent particles carrying multiple antigens, which allows simultaneous immunization against multiple pathogens.

## EXAMPLES

### EXAMPLE 1

#### 20 Construction of Vectors and Method of Plant Transformation

Construction of Plasmids containing recombinant TMV carrying chimeric AlMV CP Tobacco mosaic virus was used as a vector for the expression of chimeric genes. The plasmids B30Rz and AvB30Rz (Proc. Natl. Acad. Sci. U.S. 88, 7204 (1991))  
25 containing the TMV genome and multiple cloning sites were a gift from Dr. William Dawson of Florida University. All

-20-

fusion capsid proteins were made using AlMV CP where the first AUG codon (start codon for *in vivo* translation of AlMV CP) was exchanged for TCG to create an *Xho*I (CTCGAG) site for cloning and an RNA molecule defective in translation (an RNA molecule which does not have a continuous open reading frame that will support the synthesis of stable and detectible polypeptide *in vivo* or *in vitro*, pSP $\Delta$ AUG, (Yusibov and Loesch-fries, Virology 208, 405 (1994)). For example, Peptides or proteins from human immunodeficiency virus (HIV) and from rabies virus were engineered as fusion with capsid protein of AlMV and cloned into B30Rz.

Construction of fusion protein consisting of full length AlMV CP and V3 loop of HIV-1MN strain

A plasmid DNA containing sequences for HIV-1 envelope protein (*env*, 160) was used as a template for the polymerase chain reaction (PCR). (Such a plasmid can be made by cloning the cDNA of the HIV-1 MN strain containing gp120 sequences including the V3 loop by using PCR cloning into the PCRII vector (Invitrogen, Inc.) or other appropriate vector. The plasmid used here was supplied by David Weiner of U. Pennsylvania). PCR on the plasmid DNA containing sequences for HIV-1 envelope protein was performed using -

5'-AGATCTCGAGATGAGTTCATCTGTAGAAATTAATTGTACA-3' as the first strand- and 5'-CGGCTCGAGCTACTAATGTTACAATG-3' as the second strand primers. The PCR products were digested by *Xho*I and ligated into pSPCP $\Delta$ AUG linearized by *Xho*I. The ligation product, pSPCPMNV3, contained the DNA coding for the HIV V3

-21-

loop and full length AlMV CP. The translation initiation codon (AUG) was created upstream of the first codon (UGC, which codes for Cys) of the V3 loop so that the full length fusion protein will be read only from this codon. The clone also contained 5'- (37 nucleotides upstream of wild type AlMV CP translation start codon) and 3'- (192 nucleotides following AlMV CP stop codon containing AlMV origin of assembly) noncoding regions of AlMV CP. The segment of pSPCPMNV3 containing the DNA for HIV-1 V3 loop, the AlMV CP, and the 5'- and 3'-noncoding regions of AlMV CP, were excised by *EcoRI* and *SmaI* then ligated into B30Rz, that had been cleaved by *XhoI*, by blunt end ligation. The resulting plasmid was pBRzCPMNV3.

This strategy (described for the cloning of V3 loop of HIV-1MN strain) was used to clone the V3 loop, *vpr* and *vpu*, of the HIV-1 NL 4.3 strain. The primers used in PCR reactions to obtain a specific sequences of these genes are listed in Table. 1. The PCR products were cloned into PSPAAUG linearized by *XhoI* to fuse with AlMV CP and create chimeric protein. The resulting plasmids were named pSPCPNLV3, pSPCPNLVpr, and pSPCPNLVpu. The full length fusion protein carrying V3 loop, *vpr*, or *vpu* was introduced into B30Rz to generate plasmids, pBRzCPNLV3, pBRzCPNLVpr, and pBRzCPNLVpu. These plasmids contain full length TMV molecule and engineered fusion proteins subcloned under the subgenomic promoter of TMV CP.

Table 1

HIV-1 NL 4.3 V3 loop: 5' primer

AGA TCT CGA GAT GAG TTC ATC TGT AGA AAT TAA TTG TACA (SEQ ID NO:5)

5 HIV-1 NL 4.3 V3 loop: 3' primer

CGG CTC GAG CTA CTA ATG TTA CAA TG (SEQ ID NO:6)

HIV-1 NL 4.3 Vpr: 5' primer

GCA CTC GAG CAG ATG GAA CAA GCC CCA (SEQ ID NO:7)

HIV-1 NL 4.3 Vpr: 3' primer:

10 GCA CTC GAG GCG GAT CTA ATG GCT CCA TT (SEQ ID NO:8)

HIV-1 NL 4.3 Vpu: 5' primer

GCA CTC GAG GTG ATG CAA CCT ATA ATA GTA (SEQ ID NO:9)

HIV-1 NL 4.3 Vpu: 3' primer:

GCA CTC GAG GCC AGA TCA TCA ATA TCC CA (SEQ ID NO:10)

15 31 DNV10C primers for rabies N protein (NV10c) and synthetic epitope (31D) presented as DNV10c:

31DNV10C: 5' primer

GCGCTCGAGATGTCCGCCGTCTACACCCGAATTATGATGAACGGAGGACGACTTAAGCG  
ATACGAGGCAGCTGAAC (SEQ ID NO:11)

20 31DNV10C: 3' primer;

GCGCTCGAGTCGTCTGCTAGTGCCACGTCGGTAAGGGTAAGTTCAGCTGCCTCGTATCG  
CTTAAGTCGTCC (SEQ ID NO:12)



-23-

31DG24 primers for linear epitope of rabies G protein (rg24) and synthetic peptide (31D) presented as DRG24:

31DG24: 5' primer

GCGCTCGAGATGTCCGCCGTCTACACCCGAATTATGATGAACGGAGGACGACTTAAGCG  
5 ACCACCAGACCAGCTTG (SEQ ID NO:13)

31DG24: 3' primer;

GCGCTCGAGTCCTCTTCCACCACAAGGTGCTCATTTTCGTCGTGAAGGT  
TCACAAGCTGGTCTGGTGGTCGCTTAAGTCGTCC (SEQ ID NO:14)

Construction of fusion proteins consisting of full length AIMV  
10 CP and chimeric rabies epitopes: DNV10c and Drg24

DNV10c is a chimera of the linear epitope (NV10c) of rabies nucleocapsid protein and the synthetic peptide 31D. Drg24 is a chimera of the linear epitope (rg24) of rabies glycoprotein and the synthetic peptide 31D. NV10c and rg24  
15 are the B cell determinants. Synthetic peptide 31D is the T cell determinant.

The linear epitopes of the N (NV10C) and G (g24) proteins of rabies virus were engineered as a chimeras with the synthetic peptide 31D and the chimeras fused with AIMV CP.  
20 Each chimera (DNV10c and Drg24) was synthesized by PCR using overlapping primers (Table 1) which serve as a template for each other. The primers are made in a way that the first strand and second strand primers has 18 homologous nucleotides that will anneal during PCR reaction. Thus each primer will  
25 serve as a template for other one and support the synthesis of new chain. The primers were created to synthesize known

-24-

amino acid sequence. The PCR products resulting from these reactions were digested by *Xho*I and cloned into pSPCPΔAUG to fuse with AIMV CP. The resulting plasmids were named as pSPCPDNV10c and pSPCPDrg24. The sequences for full length fusion capsid proteins and for 5'-3' noncoding regions were cut by *Eco*RI plus *Sma*I and cloned into B30Rz linearized by *Xho*I and subsequent blunt end ligation to create pBRzCPDNV10c, pBRzCPDrg24. All clones, described above (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPDNV10c, and pSPCPDrg24), were subjected to *in vitro* translation and sequencing analysis before they were cloned into final vector (B30Rz).

#### Construction of chimeric ACP/TMV.

To engineer a chimeric TMV encapsidated with AIMV CP we used the TMV vector (Av/TMV) that had a nontranslatable coat protein. The Av/TMV was created in the laboratory of William Dawson of Florida University. The plasmid was derived from B30Rz used for the cloning described above. The schematic representation of the plasmid is given on Fig. 1A. To engineer the chimeric virus the wild type or recombinant AIMV CP (CP carrying Drg24) was cloned so as to be under the control of TMV CP subgenomic promoter. The AIMV CP was excised from pSP65A4 (Yusibov and Loesch-Fries, Proc. Natl. Acad. Sci. US 92, 8980 (1995)) by *Eco*RI plus *Sma*I and ligated into Av/TMV digested by *Xho*I by blunt ends to create the pAv/ACP. The pAv/ACPDrg24 was created identical to pBRzCPDrg24 using the primers described above and *Xho*I cloning site.

*In vitro* transcription and translation. *In vitro* transcripts of recombinant genes or recombinant TMV were synthesized using Promega T7 or SP6 RNA polymerase and CsCl purified plasmid DNA. The reaction was performed according to manufacturer guidelines. Transcripts were capped using RNA cap structure analog [m7G(5)ppp(5)G, Biolabs]. The transcripts were assayed by *in vitro* translation to determine the messenger activity of each RNA.

*In vitro* translation reactions were performed using a wheat germ cell-free translation system (Promega) and <sup>35</sup>SMet (DuPont). The reactions were carried out as described by the manufacturer and the resulting products were separated by electrophoresis in a 13% SDS-polyacrylamide gel followed by autoradiography.

#### Preparation, inoculation and immunoassays of protoplasts

Protoplasts were isolated from axenic tobacco plants (*Nicotiana tabacum* var. *Xanthi-nc*) as described (Yusibov and Loesch-Fries, 1996) and inoculated with 3 µg of recombinant TMV transcripts per 1x10<sup>5</sup> protoplasts using a polyethylene glycol procedure (Yusibov and Loesch-Fries, Proc. Natl. Acad. Sci. U.S. 1995). After inoculation the protoplasts were incubated on ice (15 minute) pelleted and washed twice with 10% mannitol. After final spin the protoplasts were resuspended in 1xAIOK medium (0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM KNO<sub>3</sub>, 1mM MgSO<sub>4</sub>, 1 µM KI, 0.1 µM CuSO<sub>4</sub>, 10 mM CaCl<sub>2</sub> .2H<sub>2</sub>O, pH 6.5) and incubated at low light conditions at 25-27° C. The protoplasts were collected 24 hr after inoculation and assayed by

immunofluorescence using monoclonal antibodies to ALMV CP (Loesch-Fries & T. Hall, J. Gen. Virol., 47, 323 (1980)) to determine the accumulation of recombinant protein (immunofluorescent microscopy or Western analysis).

5 Immunoprecipitation of particles.

The particles extracted from plant tissue which were coinfectd with transcripts of recombinant virus were immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24). Antibodies (Dietzschold  
10 et al., Virology 64, 3804 (1990)) were mixed with recombinant virus in a ratio 1: 500 (w:w) and incubated at 4° C with agitation for two hours. Within 2 hr the suspension of (50 µl) formalin-fixed staph A cells were add to the incubation mix and continued incubate at the same conditions for one more  
15 hour. After incubation was complete the cells were pelleted and washed three time with the original buffer in which the virus particles were stored (sodium phosphate buffer, pH 7.2). The final pellet was resuspended in a 50 µl of protein loading buffer and used for the Western analysis.

20 Western analysis. Protein preparations from virus infected tissue, purified virus samples or from immunoprecipitation were separated on SDS-PAGE electrophoresis and electroblotted on to nylon membrane using Towbins transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3) overnight at 33  
25 mA. After blocking with milk (Kierkegard) proteins were reacted with appropriate antibodies Westatin stain kit manufacturer (Sigma)).

Plant infection and virus isolation

Primary infection of tobacco leaves was initiated with in vitro transcription products of recombinant TMV strains, described above. Transcription products of recombinant virus were diluted 1:1 (final concentration: 15 mM) in 30 mM sodium phosphate pH 7.2 and applied to expanding tobacco leaves (growing, 3-4 week old leaves). Inoculation was effected by gentle rubbing in the presence of carborundum (320 grit; Fisher, Pittsburgh, PA) to spread the inoculum and abrade the leaf surface. Inoculum was applied after the abrasive. Inoculated *N. bentamiana* plants were isolated in a greenhouse and maintained with normal watering and fertilization. To isolate the virus particles carrying recombinant protein from locally and systemically infected leaves of tobacco they were harvested 12 days post-inoculation. The leave tissue was frozen in liquid nitrogen and ground in prechilled mortar. Ground tissue was transferred into sterile tubes containing buffer (1 ml/1 g of tissue; 0.25 M sodium phosphate, pH 7.2) and resuspended by vortexing followed by centrifugation at 10,000 rpm for 15 minutes. All manipulations with samples were performed at 0 to 4° C. Upon centrifugation the supernatant was transferred into new tubes and virus particles were selectively precipitated in a buffer containing 4% polyethylene glycol (MW 15,000 - 20,000) and 50 mM NaCl for 2 hours. Polyethylene glycol is a component that precipitates virus particles. Then virus particles were pelleted at 10,000 rpm for 20 minutes. The pellet was resuspended in a 25 mM sodium phosphate buffer pH 7.2 and centrifuged once again

under a similar conditions to separate possible plant debris and insoluble plant components. The supernatant (which contains virus) from this step was used for future experiments.

5

## Example 2

### Synthesis of fusion capsid proteins

#### In vitro translation of fusion capsid proteins

Before cloning into the final vector (30BRZ) the recombinant genes were tested for the presence of a complete open reading frame of fusion capsid proteins by sequencing and/or by *in vitro* translation. Sequence analysis was performed using CsCl purified plasmid DNA containing original PCR fragments (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPD10c, and pSPCPDrg24) and SP6 primer. CsCl purified recombinant plasmid (pSPCPMNV3, pSPCPNLV3, pSPCPNLVpr, pSPCPNLVpu, pSPCPD10c, and pSPCPDrg24). DNA containing engineered genes was digested by *Sma*I and used for the *in vitro* transcription. The capped transcripts of recombinant genes were synthesized using SP6 polymerase and translated in a wheat germ cell free translation system as described above. All tested transcripts had a messenger activity and directed the incorporation of <sup>35</sup>SMet into polypeptides of expected size.

### Translation of fusion capsid proteins in infected tobacco protoplasts

To assess the expression of fusion capsid proteins from TMV vector, the full length capped transcripts of recombinant virus were made and used for infection of tobacco protoplasts. 24 hr after inoculation with 3  $\mu$ g of transcripts per  $1 \times 10^5$ , the protoplasts were collected and used for immunoassay and for the Western analysis. Immunofluorescent assay of fixed protoplasts where we used antibodies (Loesch-Fries and T. Hall, J. Gen. Virol., 47, 323 (1980) against AlMV CP for detection showed a significant amount of protein accumulation in an individual infected cell (Data not shown). To assess the size of expressed proteins and their reaction with specific antibodies the proteins were separated on SDS polyacrylamide gel, transferred to a nylon membrane, and reacted with the monoclonal antibodies to each peptide (results not shown) or to the AlMV CP (Fig. 2). All fusion capsid proteins migrated in a range of expected size (28-35 kDa) and reacted with monoclonal antibodies to the AlMV CP or to specific peptides. The difference in the size of fusion proteins is dictated by the difference in the size of each fused with AlMV CP peptide.

### Expression of fusion capsid protein in infected plants

To assess the expression of recombinant protein in locally and systemically infected plant tissues the expanding leaves of tobacco were inoculated with transcripts of recombinant TMV. Twelve days after inoculation, the virus was purified from locally and systemically infected leaves

separately. In local infections, infection occurred in originally inoculated leaves. In systemic infection, the spread of virus was throughout the plant into new growing noninoculated leaves. Prior to purification 30-50 mg of infected tissue was used to determine if, together with TMV particles, the recombinant AlMV particles were assembled. The tissue was homogenized and the sap from it was applied on a carbon coated grid. The electron micrograph shows that spherical particles (presenting recombinant AlMV) were assembled upon infection with transcripts from all constructs (pBRzCPMNV3, pBRzCPNLV3, pBRzCPNLVpr, pBRzCPNLVpu, pBRzCPDNV10c, and pBRzCPDrg24). Fig.3 presents the results of negative staining of particles (wild type B30Rz and recombinant BRzCPDrg24) using 2% urea acetate.

Western blot analysis of purified virus samples demonstrated the presence of fusion capsid proteins in samples from both locally and systemically infected leaves (Fig. 4). This indicated that the recombinant virus was viable and it retained the fusion capsid protein during systemic movement through the plant.

#### Infection of tobacco plants with pAv/ACP and pAv/ACPDrg24.

The *in vitro* transcripts of pAv/ACP or pAv/ACPDrg24 were used for inoculation of tobacco plants. Within eight days the tissue samples were collected to assess the systemic spread of virus in noninoculated leaves. Western analysis (data not shown) detected the wt (ACP) or recombinant (ACPDrg24) protein in a systemic noninoculated leaves of tobacco indicating that



-31-

AIMV CP supported the systemic spread of CP defective TMV. The plants inoculated with transcripts of the vector itself (pAv/TMV) did not show any systemic symptoms on tobacco plants even 20 days after inoculation.

5 Assembly of recombinant AlMV particles presenting epitopes from different pathogens

Samples of infected leaf tissues from pBRzCPMNV3, pBRzCPNLV3, pBRzCPDNV10c, and pBRzCPDrg24-infected plants were taken, combined and used for the infection of tobacco plants.

10 The virus particles were purified 12 days after inoculation and assessed for co-assembly of recombinant AlMV CPs from different constructs. The virus particles were immunoprecipitated using monoclonal antibodies to the linear epitope of rabies G protein (rg24) and formalin fixed Staph

15 A cells. Immunoprecipitation products were separated by SDS polyacrylamide gel electrophoresis (above) and used for the Western blot analysis. The separated proteins were reacted to the monoclonal antibodies for the AlMV CP, antibodies against the linear epitopes of rabies N and G proteins, and

20 antibodies against V3 loop of HIV1 MN strain (National Institute of Allergy and Infectious Diseases. AIDS Research and Reference Reagent Program. #1728 Antibody to HIV-1 V3). All antibodies reacted with the immunoprecipitation product after gel separation (Fig. 5) indicating that upon co-

25 infection AlMV CP molecules will assemble into multivalent particles presenting antigenic epitopes from different pathogens. The larger molecular weight bands seen in Fig. 5

-32-

represent protein dimers similar to those of control virus (AlMV CP).

### Example 3

Immunization of mice with AlMV/TMV construct expressing the Drg24 peptide epitope of rabies glycoprotein

Eight-week old female Swiss-Webster, outbred mice were immunized with 10  $\mu$ g per dose of recombinant TMV virus engineered to express the rg24 epitope of rabies glycoprotein (Drg24-A/TMV). Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks each with and without complete Freund's adjuvant (CFA) at a 1:1, vol:vol ratio. An equal quantity of a mixture of wild type AlMV plus TMV was used with and without CFA as controls. Ten-to-fourteen days after each immunization, serum samples were obtained from individual mice and rabies virus-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA). ELISA plates (Nunc Polysorp, Denmark) were coated with 100  $\mu$ l per well of inactivated ERA-strain rabies virus (5  $\mu$ g/ml in Phosphate-buffered saline) overnight at room temperature (RT; about 25 °C). Coated plates were washed 3x with PBS-Tween (0.05%) and then blocked with 5% dried milk in PBS at RT for at least 1 hour. A series of dilutions of sera were added to the plates (30  $\mu$ l/well) for 2 to 4 hours at RT. The plates were then washed 3x with PBS-Tween and peroxidase-conjugated secondary antibodies (goat

-33-

anti-mouse IgG, either whole molecule or gamma chain specific) were added (100  $\mu$ l per well) at a final dilution of 1:2000 in PBS, for 1 hour at RT. Plates were then washed 5x with PBS-Tween and TMB substrate added (100  $\mu$ l/well) in phosphate-citrate buffer containing urea, for 30 min at RT in the dark. The reaction was stopped with 2M  $H_2SO_4$  (50  $\mu$ l per well) and the color change resulting from bound specific antibody measured at 450 nm in an ELISA plate-reader (Bio-Tek, Winooski VT). The results, expressed in O.D. units, are shown (Fig. 6A). Eighty percent of mice immunized with particles carrying Drg24 had rabies-specific antibodies (Fig. 7). Specific neutralization of rabies virus was assessed using a modified rapid fluorescent focus forming assay (Fig. 6B). Serum was inactivated by treatment for 30 minutes at 56°C and diluted in MEM medium supplemented with 10% fetal bovine serum (FBS) to a starting dilution of 1/5. The 1/5 serum dilution was further diluted serially 1/2 (1 volume plus 1 volume diluent) in 96 well plates (Nunc) such that each well contained 50  $\mu$ l of the titrated serum. Thirty  $\mu$ l of a preparation of rabies CVS-11 virus was added to each well. The rabies virus solution was prepared such that 30  $\mu$ l diluted with 50  $\mu$ l of medium and 30  $\mu$ l of BHK indicator cells ( $1.5 \times 10^6$ /ml) contained sufficient virus to cause infection of 80 to 90% of the cells in monolayer cultures after 20 hours. The 96-well plates containing the serum dilutions and rabies virus were incubated for 1 hour at 37°C prior to being carefully mixed with 30  $\mu$ l of BHK indicator cells ( $1.5 \times 10^6$ /ml). Ten  $\mu$ l of each of these mixtures was transferred to the wells of

-34-

Terasaki plates (Nunc). The Terasaki plates were incubated for 20 hours at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The plates were then washed 3x with PBS and the cells fixed by the addition of ice-cold acetone (90%) for 20 minutes. The plates were then air dried and 5 µl of a 1/40 dilution of fluorescein-conjugated rabies virus-specific antibody (Centocor) added to each well for 40 min. at 37°C. The plates were then washed 3x with water and the percentage of infected BHK cells evaluated using a fluorescent microscope (Leitz). Fig. 6B shows the presence of neutralizing antibodies in sera.

#### Example 4

##### AlMV constructs not involving TMV nucleic acid

AlMV constructs free of TMV RNA analogous to those described above are constructed in a matter analogous to the TMV recombinant constructs. AlMV nucleic acid is substituted for the TMV RNA. The structure of the AlMV genome is published and the required functions coded for by the AlMV genome have been mapped. (Bol et al., Virology 46, 73 (1971); Bol et al., Virology 58, 101 (1974)).

#### Example 5

Immunization of mice with AIMV/TMV construct containing recombinant AIMV CP carrying chimeric rabies peptide Drg24 and challenge of immunized mice with CVS-24 strain rabies virus.

-35-

Eight week old female Swiss-Webster, outbred mice were immunized with 50 mg per dose of recombinant TMV virus engineered to express the recombinant AlMV CP (CPDrg24) carrying chimeric epitome (DRg24) of rabies virus. Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks. No adjuvant was used in this experiment. An equal quantity of a mixture of wild type AMV plus TMV was used as a control. Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers were assessed. Antigen-specific antibody analysis of serum was preformed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3.

Specific neutralization of rabies virus was assessed using a modified rapid fluorescent focus forming assay. The assay was performed as described in Example 3 using CVS-11 strain rabies virus (Table 2). Fourteen days after the third immunization the groups of mice (10 mice in a group) were challenge inoculated with a deadly dose equal to approximately ten times the IMLD50 ("intramuscular lethal dose 50") of CVS-24 strain rabies virus. All control mice (nonimmunized and mice immunized with vector only) died by day 6 to 7 while 40% of CPDrg24 immunized mice were protected. 60% of CPDrg24 immunized mice died by day 15-16. However, all immunized mice survived longer than either the control or nonimmunized mice, indicating some level of protective immunity for all immunized

mice. The results of the challenge experiment are presented in Table 2.

#### Example 6

Immunization of mice with an AlMV/TMV construct  
5 containing recombinant AlMV CP carrying the V3 loop of HIV-1  
MN strain.

Eight week old female Swiss-Webster, outbred mice were immunized with 10 mg per dose of recombinant TMV virus engineered to express the recombinant AlMV CP (CPMNV3)  
10 carrying V3 loop of HIV-1 MN strain. Seven immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks. Three immunizations of 0.1 ml were administered intra-peritoneally at intervals of 2 weeks each with and without complete Freund's adjuvant (CFA) at 1:1, vol:vol  
15 ratio. An equal quantity of a mixture of wild type AMV plus TMV (30B/AlMV in Fig. 8) was used with and without CFA as a control. Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers were assessed. Antigen-specific antibody  
20 analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3. Sera from mice immunized with CPMNV3 were assessed for the presence of antibodies specific for the synthetic peptide derived from the V3 loop of HIV-1. Low levels of serum  
25 antibodies specific for HIV were detectable after the third inoculation. Fourteen days after the last immunization with

-37-

CPMNV3 serum antibodies specific for the V3 loop of the HIV-1 MN isolate were detected in both ELISA and neutralization assays as shown in Figure 8A, 8B, and 8C. While preimmune sera had low neutralizing activity (about 25%) at dilutions 1:128 and 1:256, sera from the experimental mice inoculated with CPMNV3 demonstrated, on average, approximately 80% and 76% neutralizing activity at the same dilutions respectively (Fig. 8C).

The HTLV-I/MT2 cell lines (obtained from the NIH AIDS Research and References Reagent Program) were used as target cells in the HIV-1 neutralization assay. These cells were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin and pyruvate. The cell-free HIV-1/MN isolate was propagated in HTLV-1/MT2 cells as described. Cell-free virus (100 TCID<sub>50</sub>) was preincubated with different dilutions of heat-inactivated preimmune or immune sera for 1 hr at 37° C. Following incubation, the serum-treated virus was used to infect HTLV-I/MT2 cells. Syncytia formation was evaluated 5 days after inoculation of HTLV-I/MT2 cells by phase contrast microscopy.

#### Example 7

Oral immunization of mice with AMV/TMV construct containing recombinant AIMVCP carrying chimeric rabies peptide Drg24.

-38-

Eight week old female Swiss-Webster, outbred mice were immunized via gastric intubation with 250 mg per dose of recombinant TMV virus engineered to express the AlMV CP (CPDrg24) carrying chimeric epitope (Drg24) of rabies virus.

5 Five immunizations of 0.1 ml were administered orally at intervals of 2 weeks. No adjuvant was used in this experiment. An equal quantity of a mixture of wild type AMV plus TMV (A/TMV in Fig. 9A and 9B) was used as a control.

10 Ten-14 days after each immunization serum samples were obtained from individual mice and rabies virus-specific antibody titers assessed. Antigen-specific antibody analysis of serum was performed using a solid phase enzyme-linked immunoabsorbant assay (ELISA) as described in Example 3. The increasing levels of rabies virus-specific IgG and IgA were

15 detected in the sera of immunized mice (Figs. 9A and 9B).



Table 2

Neutralization titers of sera from the mice immunized with CPDrG24 and challenge infection of these mice with CVS-24 strain rabies virus.

5	Groups of mice	Neutralization titers (mean titer)	Challenge with CVS-24 Strain Rabies Virus			
			Days after challenge (dead/total)			SURVIVALS
			6-7	10-11	15-16	
10	Mice immunized with CPDrG24	165	0/10	5/10	6/10	4/10
	Mice immunized with A/TMV	0	10/10	0	0	0
15	Non immune mice	0	10/10	0	0	0

-40-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: Koprowski, Hilary  
Yusibov, Vidadadi .  
Hooper, Douglas C.  
Modelska, Anna
- (ii) TITLE OF INVENTION: Polypeptides Fused with Plant Virus Coat  
Proteins
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Volpe and Koenig, P.C.  
(B) STREET: 400 One Penn Center  
(C) CITY: Philadelphia  
(D) STATE: Pennsylvania  
(E) COUNTRY: USA  
(F) ZIP: 19103
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE:  
(B) COMPUTER:  
(C) OPERATING SYSTEM:  
(D) SOFTWARE:
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:  
(A) NAME:  
(B) REGISTRATION NUMBER:  
(C) REFERENCE/DOCKET NUMBER: TJU-PT004PC
- (viii) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (215) 568-6400  
(B) TELEFAX: (215) 568-6499

## (2) INFORMATION FOR SEQ ID NO:1

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear
- (iii) HYPOTHETICAL: N
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- Cys Thr Arg Pro Asp Tyr Asp Lys Arg Lys Arg Ile His Ile Gly Pro  
1 5 10 15  
Gly Arg Ala Phe Tyr Thr Thr Lys Asp Ile Ile Gly Thr Ile Arg Gln  
20 25 30  
Ala His Cys  
35

## (2) INFORMATION FOR SEQ ID NO:2

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

-41-

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Thr Arg Pro Asp Asp Asp Thr Arg Lys, Ser Ile Arg Ile Gln Arg  
 1 5 10 15  
 5 Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asp Met Arg  
 20 25 30  
 Gln Ala His Cys  
 35

(2) INFORMATION FOR SEQ ID NO:3

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Ala Val Tyr Thr Arg Ile Met Met Asp Gly Gly Arg Leu Lys  
 1 5 10 15  
 Arg Tyr Glu Ala Ala Glu Leu Thr Leu Thr Asp Val Ala Leu Ala Asp  
 20 25 30  
 20 Asp Ser

(2) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

25

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ser Ala Val Tyr Thr Arg Ile Met Met Asp Gly Gly Arg Leu Lys  
 1 5 10 15  
 30 Arg Pro Pro Asp Gln Leu Val Ala Leu His Asp Gly Ile Glu Lys Leu  
 20 25 30  
 Val Val Glu Glu Asp Ser  
 35

(2) INFORMATION FOR SEQ ID NO:5

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGA TCT CGA GAT GAG TTC ATC TGT AGA AAT TAA TTG TAC A

40

(2) INFORMATION FOR SEQ ID NO:6

-42-

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGG CTC GAG CTA CTA ATG TTA CAA TG

26

(2) INFORMATION FOR SEQ ID NO:7

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCA CTC GAG CAG ATG GAA CAA GCC CCA

27

(2) INFORMATION FOR SEQ ID NO:8

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCA CTC GAG GCG GAT CTA ATG GCT CCA TT

29

(2) INFORMATION FOR SEQ ID NO:9

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCA CTC GAG GTG ATG CAA CCT ATA ATA GTA

30

(2) INFORMATION FOR SEQ ID NO:10

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

-43-

GCA CTC GAG GCC AGA TCA TCA ATA TCC CA

29

## (2) INFORMATION FOR SEQ ID NO:11

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

10 GCGCTCGAGA TGTCCGCCGT CTACACCCGA ATTATGATGA ACGGAGGACG ACTTAAGCGA 60  
TACGAGGCAG CTGAAC 76

## (2) INFORMATION FOR SEQ ID NO:12

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

20 GCGCTCGAGT CGTCTGCTAG TGCCACGTCG GTAAGGGTAA GTTCAGCTGC CTCGTATCGC 60  
TTAAGTCGTC C 71

## (2) INFORMATION FOR SEQ ID NO:13

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

30 GCGCTCGAGA TGTCCGCCGT CTACACCCGA ATTATGATGA ACGGAGGACG ACTTAAGCGA 60  
CCACCAGACC AGCTTG 76

## (2) INFORMATION FOR SEQ ID NO:14

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

40 GCGCTCGAGT CCTCTTCCAC CACAAGGTGC TCATTTTCGT CGTGAAGGTT CACAAGCTGG 60  
TCTGGTGGTC GCTTAAGTCG TCC 83

-44-

## (2) INFORMATION FOR SEQ ID NO:15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL: N

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Glu Gln Ala Pro Glu Asp Gln Gly Pro Gln Arg Glu Pro Tyr Asp  
 1 5 10 15  
 Glu Trp Thr Leu Glu Leu Leu Glu Glu Leu Lys Ser Glu Ala Val Arg  
 20 25 30  
 His Phe Pro Arg Ile Trp Leu His Asp Leu Gly Gln His Ile Tyr Glu  
 35 40 45  
 Thr Tyr Gly Asp Thr Trp Ala Gly Val Glu Ala Ile Ile Arg Ile Leu  
 50 55 60  
 Gln Gln Leu Leu Phe Ile His Phe Arg Ile Gly Cys Arg His Ser Arg  
 65 70 75 80  
 Ile Gly Val Thr Arg Gln Arg Arg Ala Arg Asp Gly Ala Ser Arg Ser  
 85 90 95 96

## (2) INFORMATION FOR SEQ ID NO:16

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (iii) HYPOTHETICAL: N

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Glu Pro Ile Ile Val Ala Ile Val Ala Leu Val Val Ala Ile Ile  
 1 5 10 15  
 Ile Ala Ile Val Val Trp Ser Ile Val Ile Ile Glu Tyr Arg Lys Ile  
 20 25 30  
 Leu Arg Gln Arg Lys Ile Asp Arg Leu Ile Asp Arg Leu Ile Glu Arg  
 35 40 45  
 Ala Glu Asp Ser Gly Asp Glu Ser Glu Gly Glu Val Ser Ala Leu Val  
 50 55 60  
 Glu Met Gly Val Glu Met Gly His His Ala Pro Trp Asp Ile Asp Asp  
 65 70 75 80  
 Leu  
 81

\* \* \*

**CLAIMS**

1. A process of administering a polypeptide to an animal, the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed in a plant cell to produce a fusion capsid protein, said fusion protein comprising a plant virus capsid protein and a polypeptide that is not a plant virus capsid protein, said plant virus capsid protein being an AlMV capsid protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid protein; and

(3) administering the fusion capsid protein or a portion thereof to an animal.

2. The process of Claim 1 wherein the recombinant plant virus nucleic acid comprises TMV nucleic acid.

3. The process of Claim 1 wherein the result of the process is protective immunity.

4. The process of Claim 1 wherein in step (3) the fusion capsid protein is part of a plant or plant material.

5. A process of Claim 1 wherein in step (3) the fusion capsid protein is extracted in purified form from the plant

and administered as a protein substantially free of other compounds of the plant.

6. A process of Claim 1 wherein the polypeptide is a rhabdovirus protein.

7. A process of Claim 1 wherein the polypeptide is a human immunodeficiency virus protein.

8. A process of Claim 12 wherein the animal is a mammal.

9. A process of claim 1, the process comprising the steps of:

(1) infecting a plant cell with a plurality of recombinant plant virus nucleic acids that will be processed in a plant cell to produce a plurality of fusion capsid proteins, each comprising a different polypeptide that is not a plant virus capsid protein, and each further comprising an AlMV capsid protein or ilarvirus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid proteins; and

(3) administering the fusion capsid proteins or a portion thereof to an animal.



10. A process for producing a polypeptide, the process comprising the steps of:

(1) infecting a plant cell with recombinant plant virus nucleic acid that will be processed by a plant cell to produce a fusion capsid protein comprising an AlMV or ilarvirus capsid protein and the polypeptide, said polypeptide not a plant virus capsid protein, thereby creating a infected cell;

(2) cultivating the infected cell, or a derivative cell derived from said infected cell, under conditions where said infected cell or derivative cell makes the fusion capsid protein.

11. A recombinant virus with a tripartite fusion capsid protein, the fusion capsid protein comprising a capsid protein of a virus that is not AlMV or an ilarvirus, a capsid protein or AlMV or an ilarvirus, and a polypeptide that is not a plant virus polypeptide.

12. The process of Claim 1 wherein the result of the process is an immune response against the polypeptide that is part of the fusion capsid protein but is not a plant virus protein.

13. The process of Claim 12 wherein the immune response is the production of antibodies against the polypeptide.

Fig. 1A

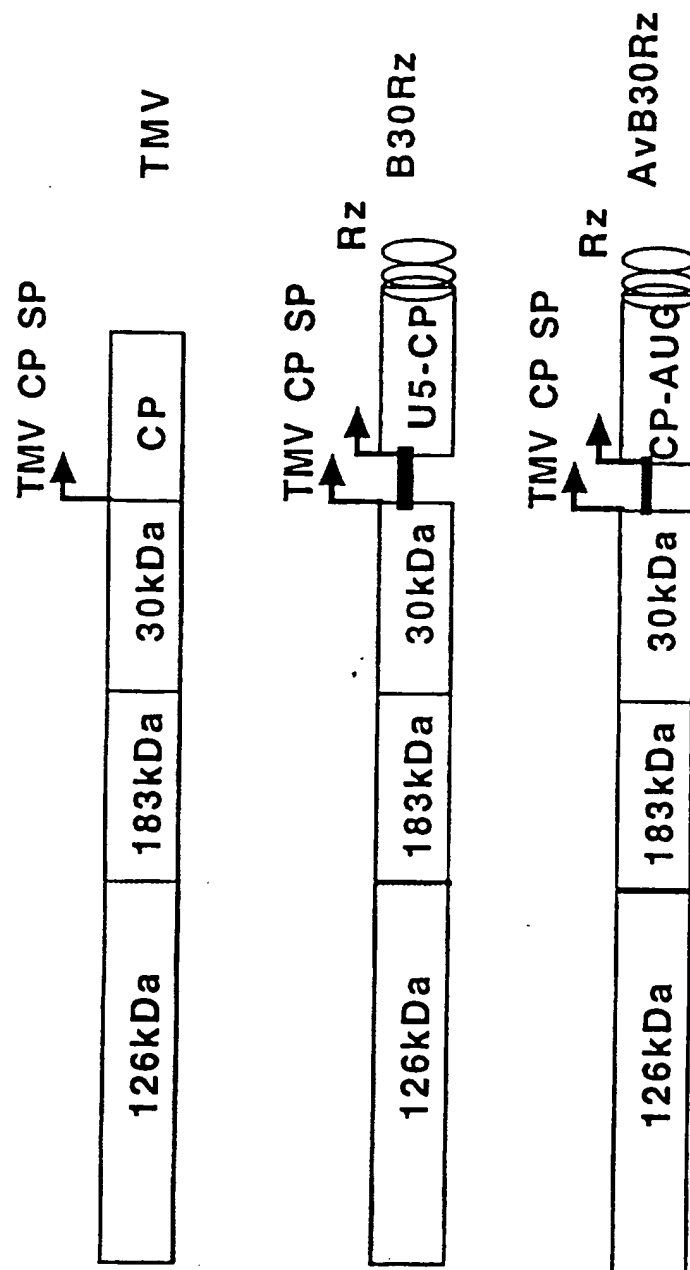


Fig. 1B

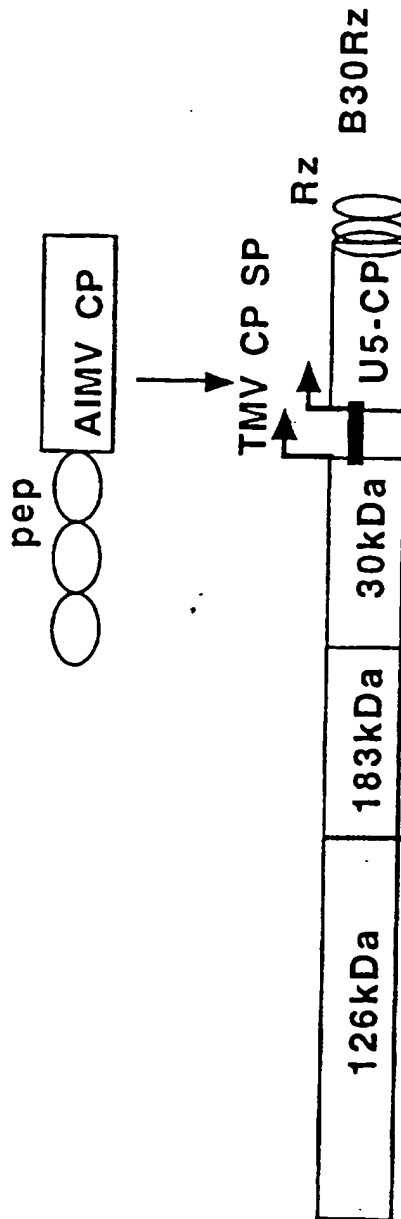


Fig. 2

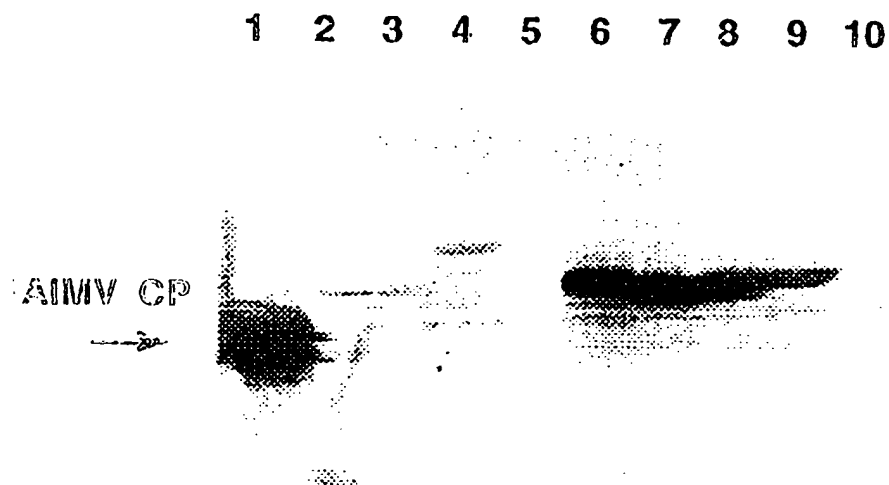


Fig. 3A

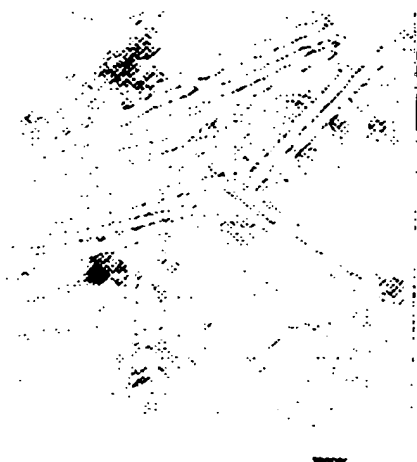


Fig. 3B



Fig. 4

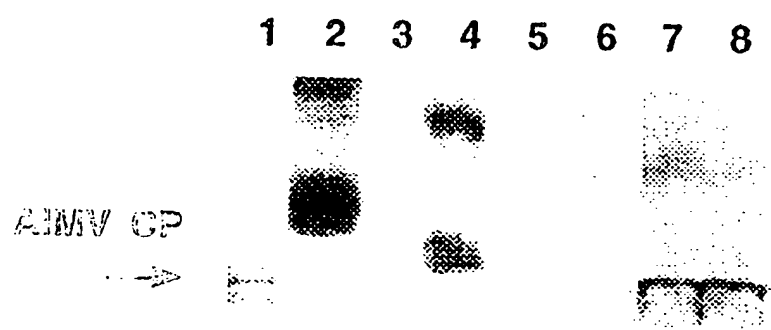


Fig. 5

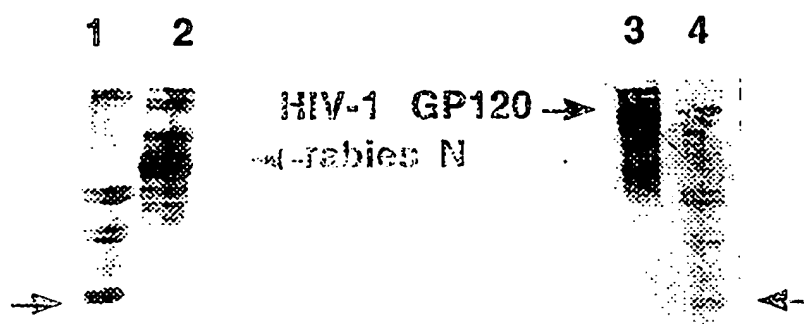


Fig. 6A

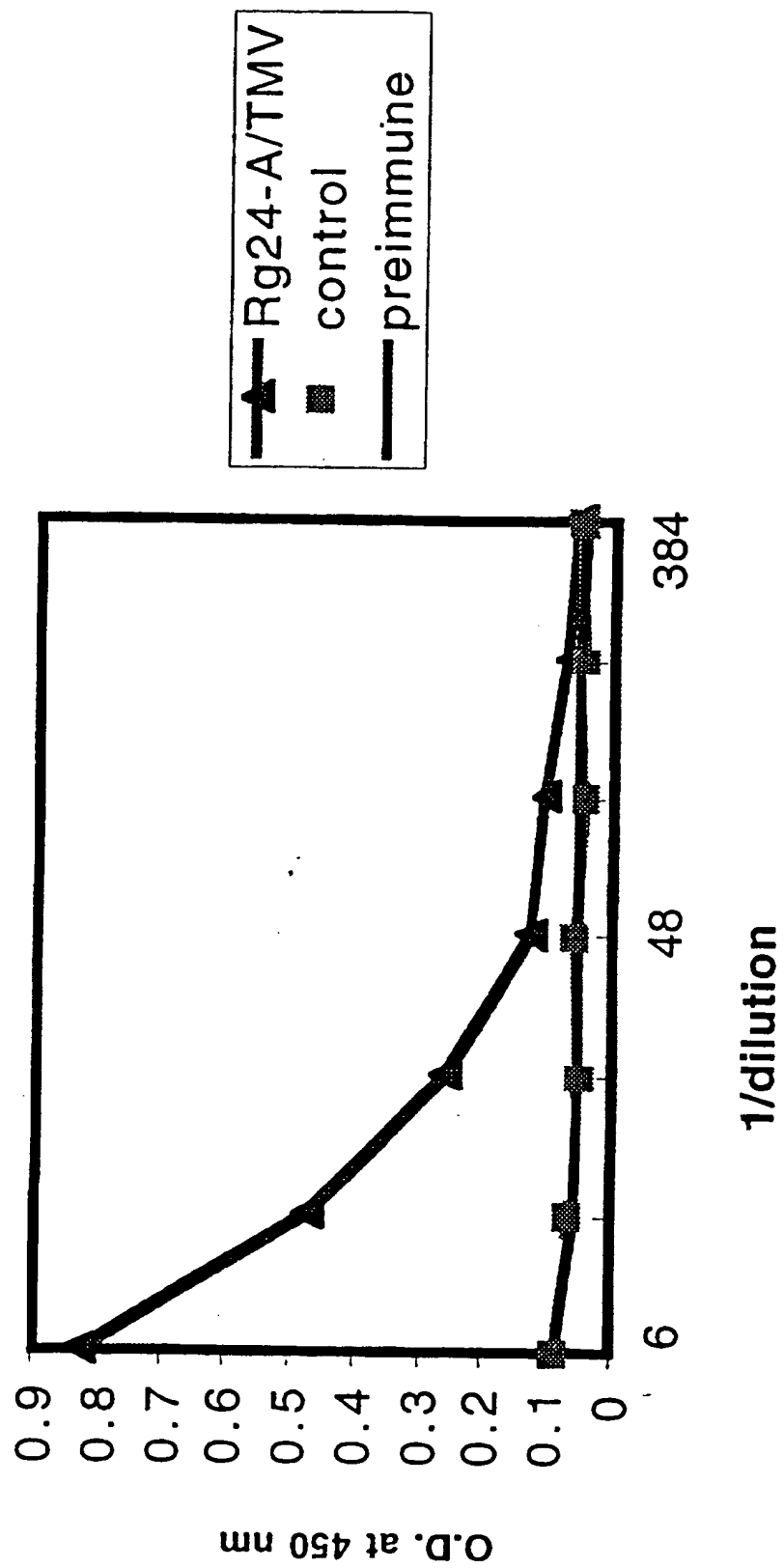




Fig. 6B

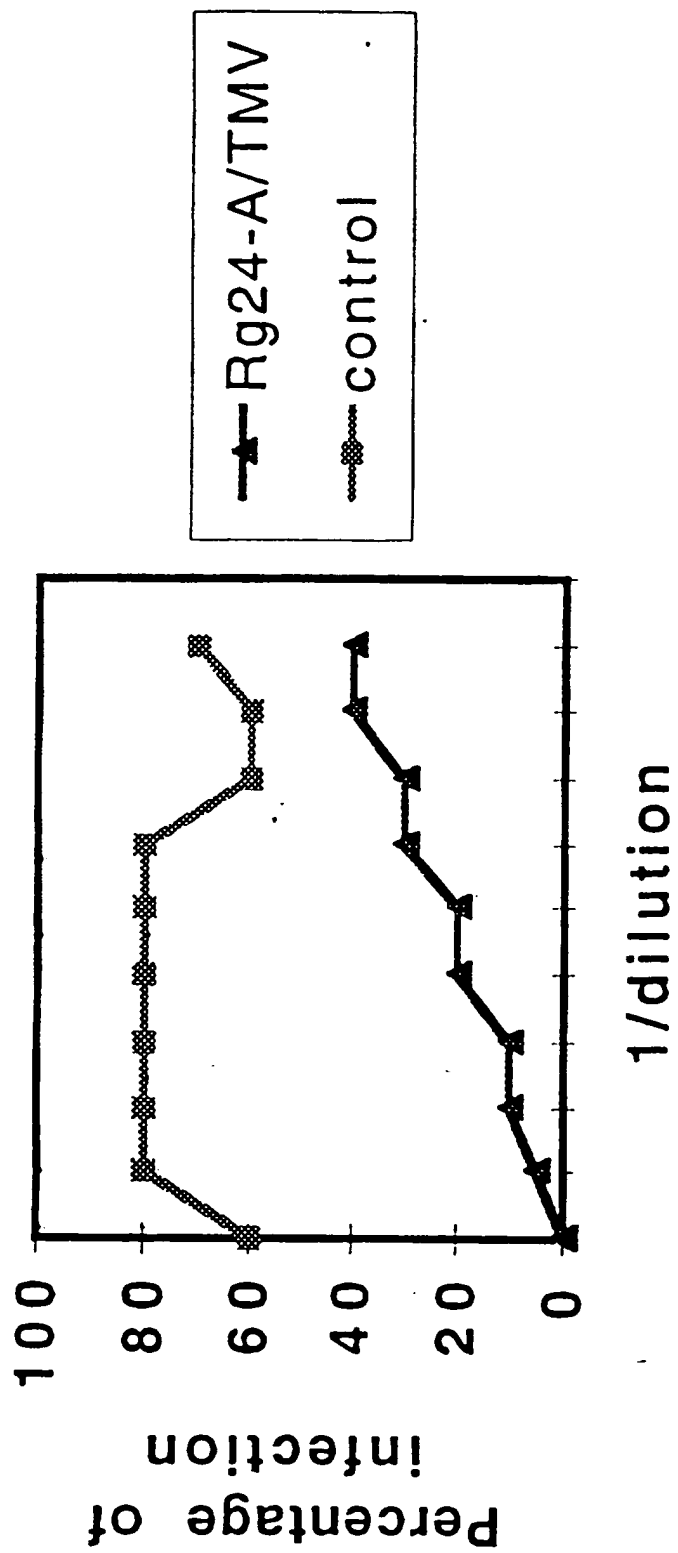


Fig. 7

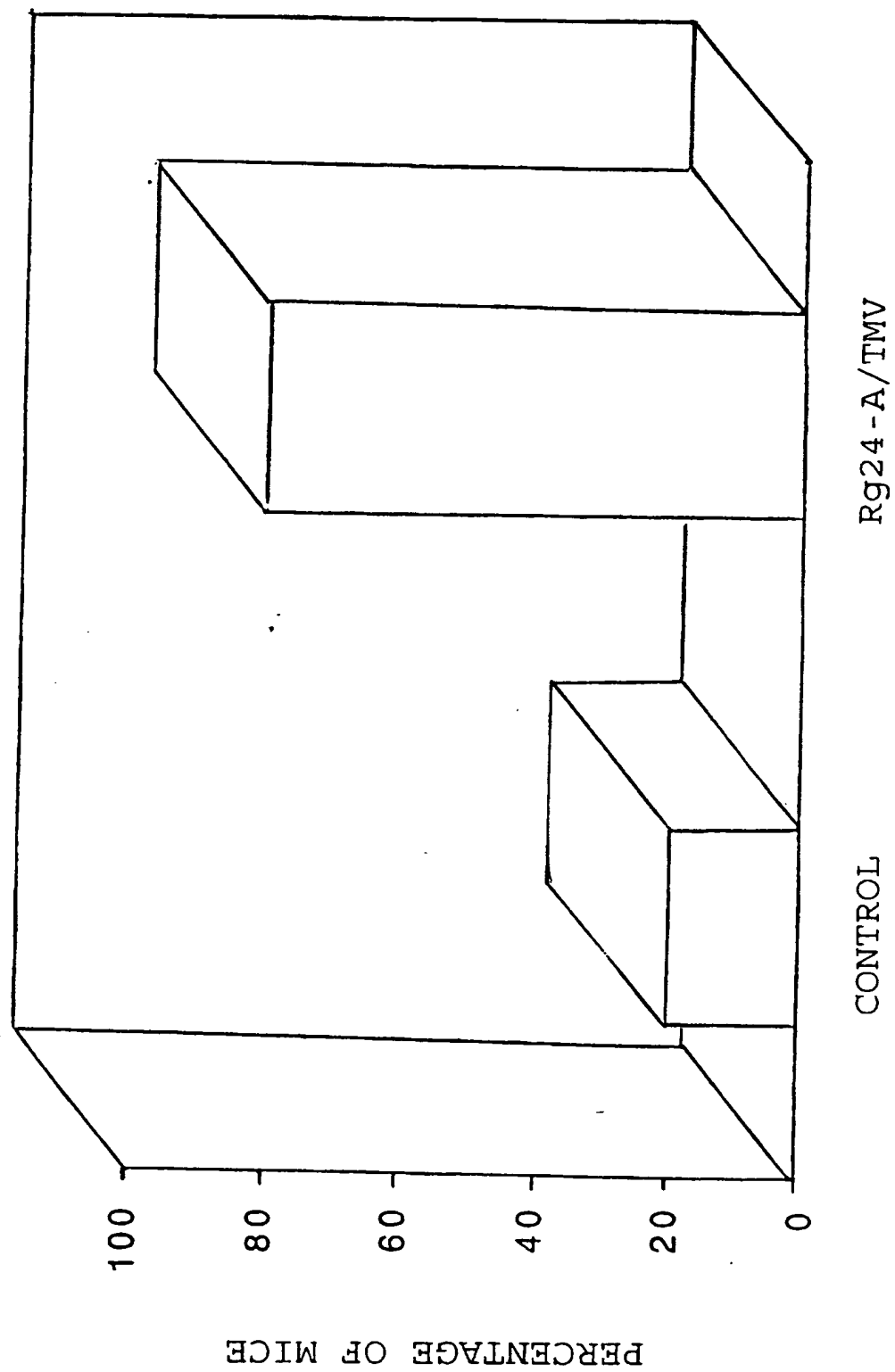


Fig. 8A

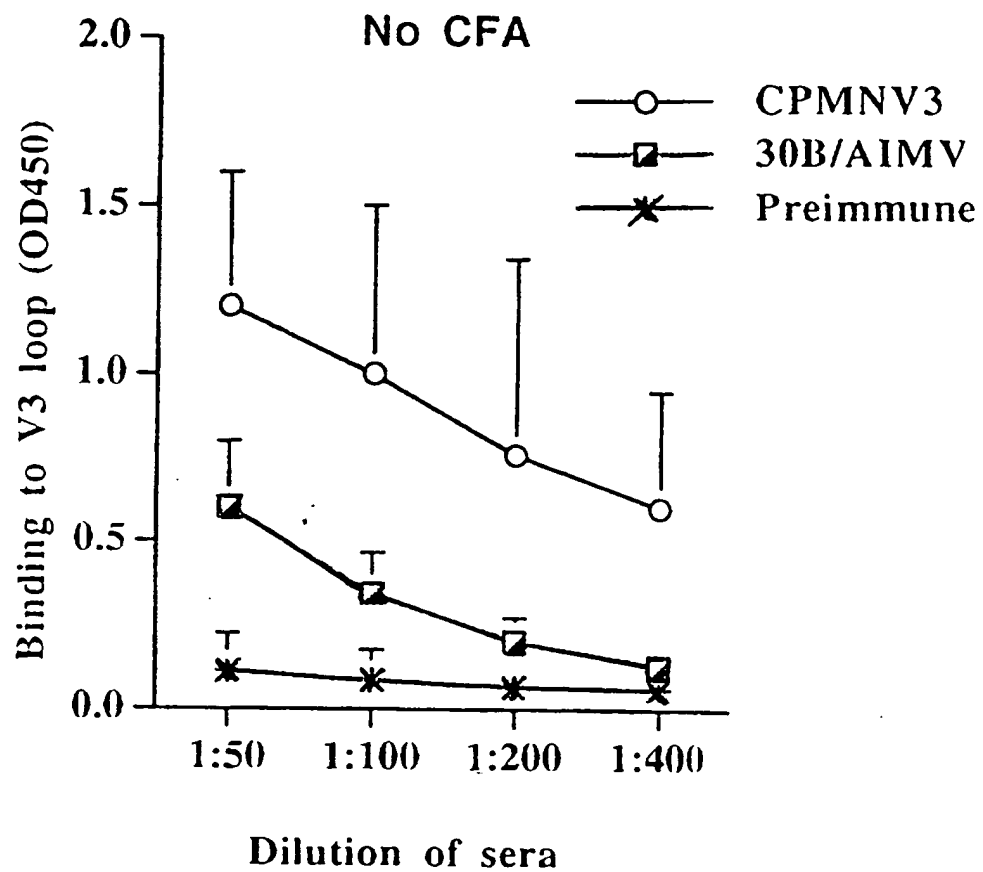


Fig. 8B

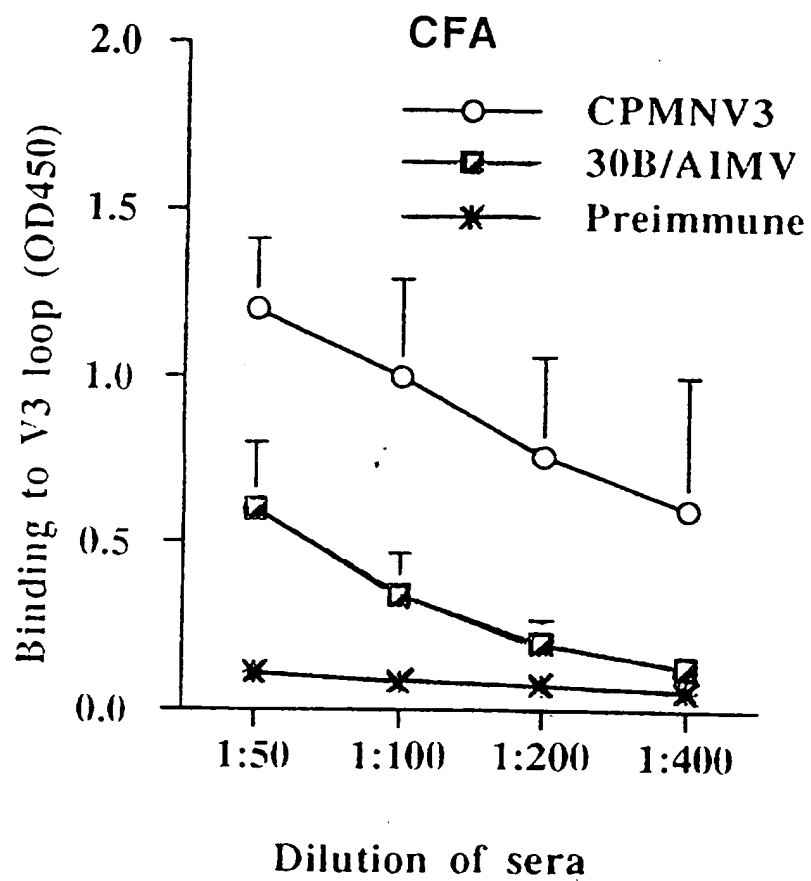


Fig. 8C

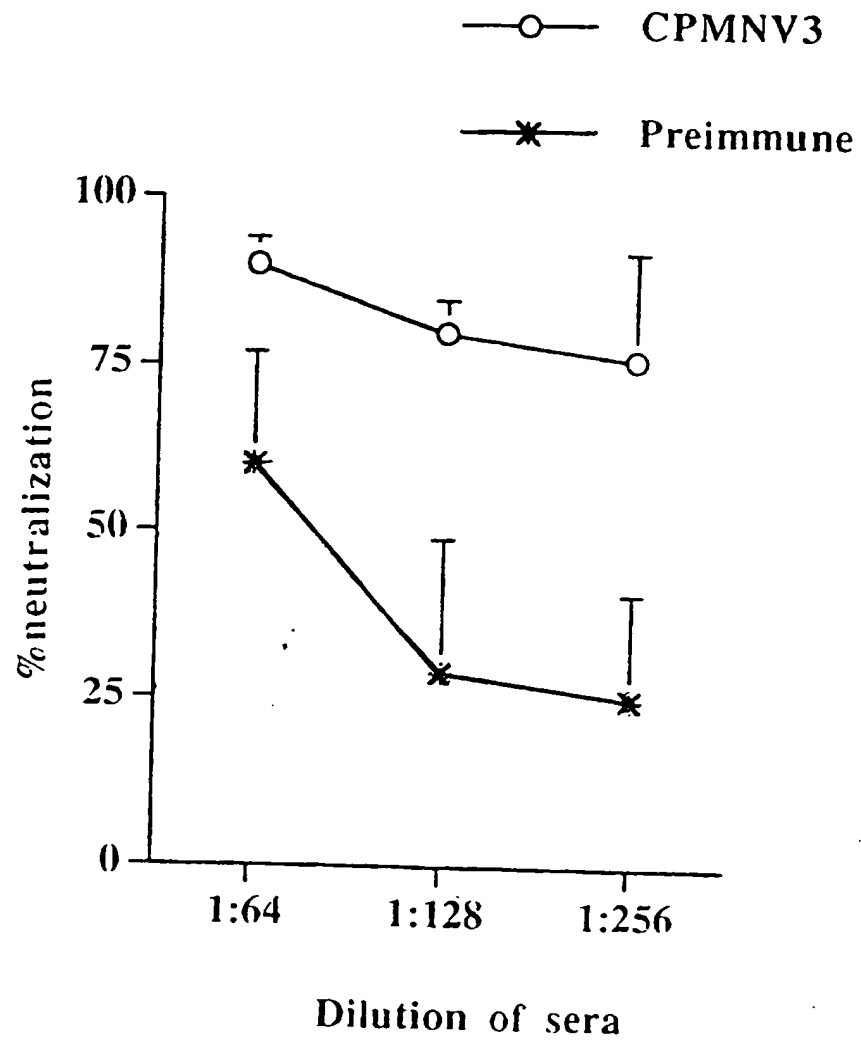


Fig. 9A

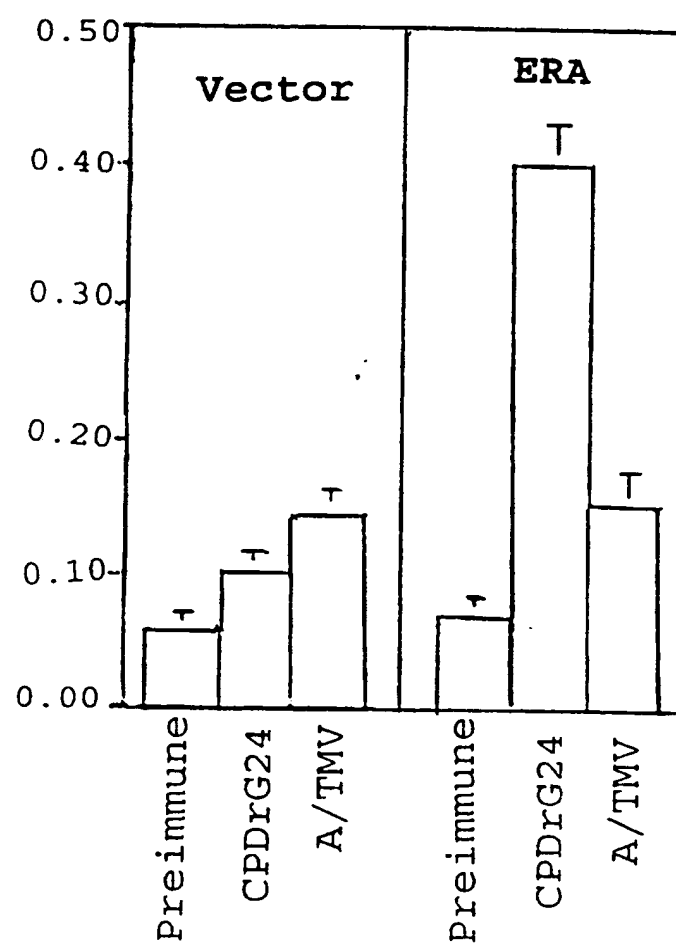
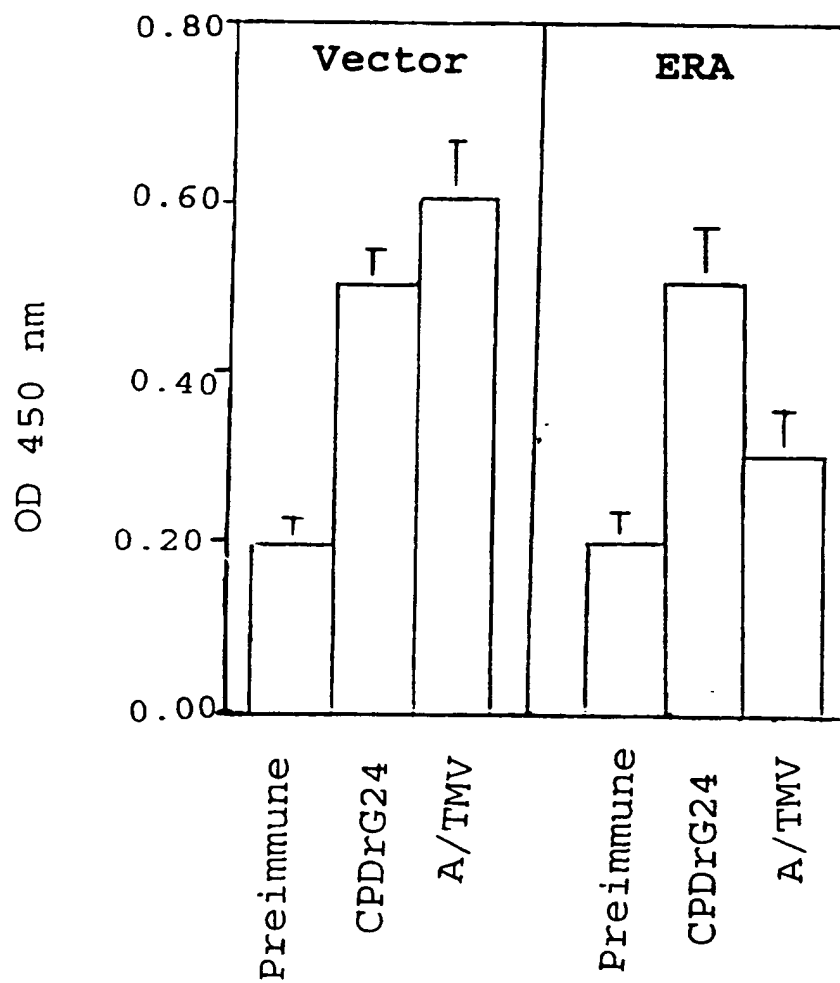


Fig. 9B



**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A01H 4/00; A61K 39/00; C07K 14/00

US CL :800/200, 205; 424/224.1, 207.1; 435/414, 419, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/200, 205; 424/224.1, 207.1; 435/414, 419, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92/18618 A1 (LOMONOSSOFF et al.) 29 October 1992, see entire article.	1-13
Y	BAER et al. Specific RNA binding by amino-terminal peptides of alfalfa mosaic virus coat protein. The EMBO Journal. 1994, Vol. 13, No. 3, pages 727-735, see Abstract.	1-13

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:		*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A*	document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B*	earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O*	document referring to an oral disclosure, use, exhibition or other means		
*P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 NOVEMBER 1997

Date of mailing of the international search report

07 JAN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PHUONG BUI

Telephone No. (703) 308-0196